Protective Effect of Ebselen, a Seleno-Organic Compound, Against the Progression of Acute Gastric Mucosal Lesions Induced by Compound 48/80, a Mast Cell Degranulator, in Rats

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ABSTRACT—The protective effect of ebselen, which possesses glutathione peroxidase-like activity and antioxidative and anti-inflammatory properties, against the progression of acute gastric mucosal lesions was examined in rats with a single intraperitoneal injection of compound 48/80 (0.75 mg/kg). Ebselen (50, 100 or 200 mg/kg) was orally administered 0.5 h after compound 48/80 treatment, at which time gastric mucosal lesions appeared. Post-administered ebselen suppressed gastric mucosal lesion progression at 3 h after compound 48/80 treatment dose-dependently, although no dose of ebselen affected the decreased gastric mucosal blood flow and increased serum serotonin and histamine concentrations found at 3 h after the treatment. A decrease in Se-glutathione peroxidase activity and increases in myeloperoxidase and xanthine oxidase activities and the concentration of thiobarbituric acid reactive substances were found in gastric mucosal tissues at 0.5 h after compound 48/80 treatment, and these changes were further enhanced at 3 h. Post-administered ebselen attenuated all these changes found at 3 h after compound 48/80 treatment dose-dependently. The present results indicate that ebselen exerts a protective effect against the progression of compound 48/80-induced acute gastric mucosal lesions in rats, and they suggest that this protective effect of ebselen could be due to its glutathione peroxidase-like activity and its antioxidative and anti-inflammatory properties.

Keywords: Compound 48/80, Gastric mucosal lesion (rat), Ebselen, Glutathione peroxidase, Neutrophil infiltration

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) is a seleno-organic compound. This seleno-organic compound is known to possess glutathione peroxidase (GSH-px)-like activity and antioxidative and anti-inflammatory properties (1 – 8). Ebselen has been reported to protect against various types of experimentally induced gastric mucosal lesions such as aspirin-, diclofenac-, HCl-, acidified ethanol-, ethanol-, water immersion restraint stress-, burn stress- and ischemia-reperfusion-induced gastric mucosal lesions (9 – 15).

Compound 48/80 is known to cause degranulation of connective tissue mast cells, but not mucosal mast cells, with release of serotonin and histamine from the cells (16 – 18). We have shown in rats with a single treatment of compound 48/80 that the development of gastric mucosal lesions occurs with decreases in Se-glutathione peroxidase (Se-GSH-px) activity and vitamin E and hexosamine contents and increases in neutrophil infiltration, xanthine oxidase (XO) activity, and lipid peroxide content in the gastric mucosal tissue and that gastric mucosal blood flow is reduced with gastric mucosal lesion formation, while the decreased blood flow is recovered with the lesion progression (19). We have also shown in rats treated once with compound 48/80 that neutrophils infiltrating into the gastric mucosal tissue participate in gastric mucosal lesion formation and progression, while the xanthine-XO system in the gastric mucosal tissue takes part mainly in the lesion progression (20). Furthermore, it has been shown in rats treated once with compound 48/80 that acutely released endogenous serotonin contributes to gastric mucosal lesion formation, while released endogenous histamine mainly contributes to the lesion progression, although gastric acid plays little role in the pathogenesis of compound 48/80-
induced gastric mucosal lesions (19, 21). Our recent report has shown that pre-administered ebselen alleviates compound 48/80-induced acute gastric mucosal lesions at the late stage, but not the early stage, of the lesion development in rats, possibly through its GSH-px-like activity and its antioxidative and anti-inflammatory properties (22). However, it is still unknown whether ebselen exerts a protective effect against the progression of acute gastric mucosal lesions in rats treated once with compound 48/80 through its GSH-px-like activity and its antioxidative and anti-inflammatory properties.

In the present study, therefore, we attempted to clarify the protective effect of ebselen against the progression of compound 48/80-induced acute gastric mucosal lesions in rats by examining the effect of ebselen administered orally at the stage of gastric mucosal lesion formation on the lesion progression and changes in the activities of gastric mucosal Se-GSH-px, myeloperoxidase (MPO), an index of tissue neutrophil infiltration (23), and XO and the content of gastric mucosal thiobarbituric acid (TBARS), an index of lipid peroxidation, with the lesion progression in rats with a single compound 48/80 treatment. We further examined the effect of the post-administered ebselen on changes in gastric mucosal blood flow and serum serotonin and histamine concentrations with gastric mucosal lesion progression in the compound 48/80-treated rats.

MATERIALS AND METHODS

Materials

Ebselen was kindly provided by Daichi Pharmaceutical Co., (Tokyo). Compound 48/80, methyl serotonin, 3,3',5,5'-tetramethylbenzidine, xanthine and yeast glutathione reductase were purchased from Sigma Chemical Co. (St. Louis, MO, USA); ethylenediaminetetraacetic acid (EDTA), reduced glutathione (GSH), NADPH, α-phthalaldehyde, 2-thiobarbituric acid and other chemicals from Wako Pure Chemicals Ind., Co. (Osaka).

Animals

Male Wistar rats aged six weeks were obtained from Nippon SLC Co. (Hamamatsu). The animals were housed in cages in a ventilated animal room with controlled temperature (23 ± 2°C) and relative humidity (55 ± 5%) and with 12 h of light (7:00 to 19:00). They were maintained on standard laboratory chow (Oriental MF; Oriental Yeast Co., Tokyo) and tap water ad libitum for one week. All animals received humane care in compliance with the guideline of the Animal Care and Use Committee of Fujita Health University.

Gastric mucosal lesion induction by compound 48/80

Compound 48/80 (0.75 mg/kg body weight), dissolved in distilled water, was intraperitoneally injected to 7-week-old rats fasted for 24 h, as described previously (19 – 22). The control rats received an intraperitoneal (i.p.) injection of an equal volume of distilled water. All animals were maintained with free access to water and without food during the experiment. The animals were sacrificed under ether anesthesia 0.5 or 3 h after compound 48/80 injection. The stomachs were removed, inflated with 10 ml of 0.9% NaCl and put into 10% formalin for 10 min. The stomachs were then opened along the greater curvature and examined for lesions in the glandular part under a dissecting microscope (×10). The severity of gastric mucosal lesions was estimated using the index of the following eight grades of lesions as described in our previous reports (19 – 22): grade 0, no lesion (normal); grade I, edema only; grade II, damaged area of 1 – 10 mm²; grade III, damaged area of 11 – 20 mm²; grade IV, damaged area of 21 – 30 mm²; grade V, damaged area of 31 – 40 mm²; grade VI, damaged area of 41 – 50 mm²; grade VII, damaged area of > 51 mm². The severity of gastric mucosal lesions was also estimated using the lesion score classification. Namely, grades 0 to VII used in the above-described lesion index were defined as scores 0 to 7, respectively.

Administration of ebselen

Ebselen was suspended in 0.5% carboxymethylcellulose sodium solution at a constant dosing volume of 5 ml/kg body weight. Ebselen (50, 100 or 200 mg/kg body weight) was orally administered to fasted rats with a stomach tube at 0.5 h after compound 48/80 treatment. Ebselen-untreated rats received an equal volume of 0.5% carboxymethylcellulose sodium solution at the same time point.

Determinations of gastric mucosal Se-GSH-px, MPO, XO and TBARS

Gastric mucosal Se-GSH-px and MPO were assayed by the methods of Hochstein and Utley (24) and Suzuki et al. (25), respectively. For assays of both enzymes, gastric mucosal tissues were homogenized in 9 vol of ice-cold 0.05 M Tris-HCl buffer (pH 7.4). After sonication on ice for 20 s using a Handy Sonic model UR-20P (Tomy Seiko Co., Tokyo), the homogenate was centrifuged at 4°C (10,000 × g, 20 min), and the resultant supernatant was dialyzed against 100 vol of the same buffer at 4°C for 24 h. Se-GSH-px activity was determined at 37°C by recording the decrease in absorbance at 340 nm following the oxidation of NADPH in the presence of H₂O₂, GSH and yeast glutathione reductase. One unit (U) of this activity is defined as the amount of enzyme oxidizing 1 μmol NADPH per min. MPO activity was assessed by measuring the H₂O₂-dependent oxidation of tetramethylbenzidine at 37°C.
One unit of this enzyme is defined as the amount of enzyme causing a change in absorbance of 1.0 per min at 655 nm. Gastric mucosal XO was assayed by the method of Hashimoto (26). For this enzyme assay, gastric mucosal tissues were homogenized in 9 vol of ice-cold 0.25 M sucrose. The homogenate was sonicated as described above. The sonicated homogenate was centrifuged at 4°C (10,000 × g, 20 min), and the resultant supernatant was dialyzed against 100 vol of the same solution at 4°C for 24 h. XO activity was assessed by measuring the increase in absorbance at 292 nm following the formation of uric acid at 30°C. One unit of this enzyme is defined as the amount of enzyme forming 1 μmol uric acid per min. Gastric mucosal TBARS was spectrophotometrically determined by the thiobarbituric acid method of Ohkawa et al. (27) except that 1.0 mM EDTA was added to the reaction medium. For this determination, gastric mucosal tissues were homogenized in 9 vol of ice-cold 20 mM EDTA. The amount of TBARS is expressed as that of malondialdehyde (MDA) equivalents. Determinations of serum serotonin and histamine: For serum serotonin and histamine determinations, blood was collected from the inferior vena cava of rats upon sacrifice and then serum was obtained from the collected blood by centrifugation. Serum samples were deproteinized by adding perchloric acid at a final concentration of 3% and then centrifuged at 4°C for 10 min (10,000 × g). Serum serotonin was measured by the method of Shibata et al. (28) using high-performance liquid chromatography with electrochemical detection except that 40 mM sodium dihydrogenphosphate used for the mobile phase was replaced by 0.1 M citric acid – 0.1 M sodium acetate (0.7:1.0, v/v). Methyl serotonin was used as an internal standard. Serum histamine was measured by the methods of Lorenz et al. (29) and Shore et al. (30). Histamine was reacted with α-phthalaldehyde and the intensity of the resultant fluorescence was measured using a spectrophotometer (the excitation wavelength, 360 nm; the emission wavelength, 450 nm).

Measurement of gastric mucosal blood flow
Gastric mucosal blood flow was measured using a laser Doppler flowmeter, Laser Flow BRL-100 (Bio Research Center Co., Nagoya), as described in our previous reports (19 – 22). Rats used for this measurement were anesthetized with pentobarbital sodium 10 min before the onset of the measurement and the abdomen was opened on an operation mat. The mat was heated at 37°C during the operation and blood flow measurement. The laser probe was attached to the serosal side of the corpus mucosa by aid of a cyanoacrylate-type instantaneous adhesive, Aron Alpha (Toha Gosei Co., Tokyo), and the blood flow changes were monitored on a recorder for at least 5 min after the onset of the measurement. Gastric mucosal blood flow in compound 48/80-treated rats is expressed as a relative percentage toward the mean value of gastric mucosal blood flow determined in control rats without compound 48/80 treatment.

Analyses of data
Results obtained for gastric mucosal and serum components and enzymes, and gastric mucosal blood flow are expressed as the mean ± S.D. The results were analyzed by a computerized statistical package (StatView). Each mean value was compared by one-way analysis of variance (one-way ANOVA) and Fisher’s PLSD (Protected Least Significance Difference) for multiple comparisons as the post hoc test. Statistical analyses of the severity of mucosal lesions were carried out using the Kruskal-Wallis test. Values of significance were set at P<0.05 for both tests.

RESULTS
Effect of post-administered ebselen on gastric mucosal lesion progression
As shown in Fig. 1 (A and B), gastric mucosal lesions appeared 0.5 h after treatment with compound 48/80 (0.75 mg/kg) and progressed at 3 h when the severity of gastric mucosal lesions was estimated using the lesion gradation or the lesion score classification. Oral administration of ebselen (50, 100 or 200 mg/kg), which was conducted 0.5 h after compound 48/80 treatment, significantly suppressed the progression of gastric mucosal lesions, and this preventive effect of ebselen occurred in a dose-dependent manner (Fig. 1: A and B). In addition, the administration of ebselen at a dose of 200 mg/kg suppressed the gastric mucosal lesion progression almost completely (Fig. 1: A and B).

Effect of post-administered ebselen on changes in serum serotonin and histamine concentrations and gastric mucosal blood
At 0.5 h after compound 48/80 treatment, serum serotonin and histamine concentrations in rats treated with compound 48/80 alone were 3.7- and 23.3-fold, respectively, higher than those in untreated control rats at 0.5 h after the treatment, while gastric mucosal blood flow in the compound 48/80-treated group was 25% of that in the control group (Fig. 2: A, B and C). At 3 h after compound 48/80 treatment, serum serotonin and histamine concentrations in the compound 48/80-treated group were 2.4- and 6.1-fold higher than those in the control group, while gastric mucosal blood flow in the compound 48/80-treated group was 75% of that in the control group (Fig. 2: A, B and C). Post-administration of ebselen (50, 100 or 200 mg/kg) did not affect the increases in serum serotonin and histamine concentrations and the decrease in gastric mu-
cosal blood flow at 3 h after compound 48/80 treatment (Fig. 2: A, B and C).

**Effect of post-administered ebselen on changes in gastric mucosal TBARS concentration and Se-GSH-px activity**

As shown in Fig. 3A, rats treated with compound 48/80 alone had a significantly higher TBARS concentration than untreated control rats at 0.5 and 3 h after the treatment; the TBARS concentrations in the former group at 0.5 and 3 h after compound 48/80 treatment were 1.1- and 1.9-fold higher than that in the latter group, respectively. Post-administration of ebselen (50, 100 or 200 mg/kg) significantly attenuated this increase in gastric mucosal TBARS concentration at 3 h after compound 48/80 treatment in a dose-dependent manner (Fig. 3A). In addition, the TBARS concentration in the compound 48/80-treated group with...
post-administration of ebselen (200 mg/kg) was near that in the control group (Fig. 3A). As shown in Fig. 3B, the compound 48/80-treated group had significantly lower gastric mucosal Se-GSH-px activity than the control group at 0.5 and 3 h after the treatment; the GSH-px activities in the former group at 0.5 and 3 h after compound 48/80 treatment were 83.6 and 43.8% of that in the latter group at 0.5 and 3 h, respectively. Post-administration of ebselen (50, 100 or 200 mg/kg) significantly attenuated the decrease in gastric mucosal Se-GSH-px activity at 3 h after compound 48/80 treatment dose-dependently (Fig. 3B). In addition, the Se-GSH-px activity in the compound 48/80-treated group with post-administration of ebselen (200 mg/kg) was near that in the control group (Fig. 3B).
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The model of acute gastric mucosal lesions in rats treated once with compound 48/80, a mast cell degranulator, has been thought to be important for clarifying the roles of ischemia-reperfusion, oxidative stress, and inflammation in the pathogenesis of gastritis in humans (19–21). In addition, recent reports (31–33) have shown that Helicobacter pylori might cause gastric mucosal inflammation, the reduction of gastric mucosal blood flow, and gastric mucosal microcirculatory disturbances through mast cell degranulation. The present study has clearly shown that in rats with a single compound 48/80 treatment, ebselen administered orally at the stage of gastric mucosal lesion formation exerts a protective effect against the lesion progression in a dose-dependent manner.

In the present study, administration of ebselen (50, 100 and 200 mg/kg) to compound 48/80-treated rats at the stage of gastric mucosal lesion formation had no effect on the increases in serum serotonin and histamine concentrations found at the stage of the lesion progression, as found in the case of the pre-administration of ebselen (22). These results suggest that post-administered ebselen protects against gastric mucosal lesion progression in rats with a single compound 48/80 treatment without affecting the changes in the blood levels of histamine and serotonin released from the connective tissue mast cells.

Pre-administered ebselen is known to prevent the development of acute gastric mucosal lesions induced by compound 48/80 without affecting both the reduction of gastric mucosal blood flow at the stage of lesion formation and the recovery of the reduced blood flow at the stage of lesion progression in rats (22). In the present study, any dose of ebselen administered to rats treated once with compound 48/80 at the stage of gastric mucosal lesion formation did not affect the recovery of reduced gastric mucosal blood flow. It has been reported that when ebselen at a dose of 10, 30 or 100 mg/kg is orally administered to normal mice, serum ebselen concentration increases dose-dependently 0.5 h after the administration and the increased serum ebselen concentration is reduced gradually thereafter (14). A marked decrease in gastric mucosal blood flow occurred 0.5 h after compound 48/80 treatment; i.e., at the stage of gastric mucosal lesion formation, and the decreased gastric mucosal blood flow was partially recovered 3 h; i.e., at the stage of the lesion progression, as described above. These findings may allow us to assume that ebselen administered orally to compound 48/80-treated rats at the stage of gastric mucosal lesion formation can achieve an amount enough to exert its pharmacological actions in the gastric mucosal tissue at the stage of the lesion progression, leading to protection against the progres-
sion of compound 48/80-induced acute gastric mucosal lesions. However, further investigation is required to confirm this assumption.

Ebselen possesses GSH-px-like and anti-lipid peroxidative activities (1–3, 5). In the present study, ebselen administered to compound 48/80-treated rats at the stage of gastric mucosal lesion formation attenuated the decreased gastric mucosal Se-GSH-px activity and the increased concentration of gastric mucosal TBARS, an index of lipid peroxidation, found at the stage of the lesion progression in a dose-dependent manner. In addition, it is known that ebselen at a concentration of 10 to 100 μg/ml inhibits in vitro lipid peroxidation induced by a water-soluble radical initiator, 2,2'-azobis(2-amidinopropane), in gastric mucosal tissue preparations from compound 48/80-treated rats in a dose-dependent manner (22).

Ebselen inhibits the adhesion and transendothelial migration of polymorphonuclear leukocytes; i.e., neutrophils, both in vitro (4) and in vivo (6–9). In the present study, ebselen administered to compound 48/80-treated rats at the stage of gastric mucosal lesion formation attenuated the increases in the activities of gastric mucosal MPO, an index of tissue neutrophil infiltration (23), and XO found at the stage of the lesion progression in a dose-dependent manner. These results suggest that post-administered ebselen could exert a protective effect against gastric mucosal lesion progression in rats with a single compound 48/80 treatment through its GSH-px-like activity and antioxidative and anti-inflammatory properties. The mechanism by which administered ebselen attenuates increased gastric mucosal XO activity in compound 48/80-treated rats has not been clarified in the present study. However, ebselen at a concentration of 10 to 100 μg/ml is known to have no effect on in vitro XO activity in gastric mucosal tissue preparations from compound 48/80-treated rats (22). Our previous report has shown that in rats with a single compound 48/80 treatment, pre-administration of anti-neutrophil antiserum or NPC 14686 (α-homophenylalanine), an inhibitor of neutrophil recruitment, attenuates increased gastric mucosal XO activity at the late stage of the development of compound 48/80-induced acute gastric mucosal lesions (19). Accordingly, these findings may allow us to think of the possibility that ebselen administered to rats with a single compound 48/80 treatment at the stage of gastric mucosal lesion formation attenuates a neutrophil-mediated increase in XO activity in the gastric mucosal tissue found at the stage of the lesion progression by inhibiting neutrophil infiltration into the gastric mucosal tissue, although the mechanism by which infiltrating neutrophils cause an increase in gastric mucosal XO activity is unclear at present.

It has been shown that ebselen at a dose of 30 to 300 mg/kg inhibits gastric acid secretion in pylorus-ligated rats (13), although there is a report showing no effect of the seleno-organic compound at a dose of 200 mg/kg on gastric acid secretion in pylorus-ligated rats (10). It has also been shown that ebselen inhibits gastric acid secretion by interfering with sulphhydryl groups of the gastric proton pump, H⁺K⁺-ATPase, in vitro (34, 35). However, our previous report has shown that pre-administration of cimetidine or famotidine, a histamine H₂ receptor antagonist, at doses enough to inhibit gastric acid secretion has no effect on the formation and progression of gastric mucosal lesions in rats with a single compound 48/80 treatment (21). Accordingly, it seems unlikely that ebselen administered to rats treated once with compound 48/80 at the stage of gastric mucosal lesion formation exerts a protective effect against the lesion progression by inhibiting gastric acid secretion.

Yasuhiro et al. (36, 37) have suggested that endogenous nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) increasing in gastric mucosal tissues, in addition to enhanced gastric mucosal lipid peroxidation and released endogenous serotonin, is involved in the pathogenesis of gastric mucosal lesions induced by repeated treatment with compound 48/80 in rats. The authors have also suggested that the deleterious role of NO during compound 48/80 treatment may be accounted for by a cytotoxic action of peroxynitrite, which is formed in the presence of NO and superoxide radical (36, 37). Ebselen is known to inhibit preferentially iNOS (38) and to scavenge peroxynitrite in vitro and ex vivo (39). It has been shown that peroxynitrite induces membrane lipid peroxidation (40). We have observed that the levels of nitrite and nitrate, which are produced by oxidation of NO, increase in the gastric mucosal tissue of rats at 3 h, but not 0.5 h, after a single treatment of compound 48/80 (0.75 mg/kg) (Y. Ohta et al., unpublished data). These findings may allow us to think of the possibility that ebselen administered to rats treated once with compound 48/80 at the stage of gastric mucosal lesion formation exerts a protective effect against the lesion progression by inhibiting increased iNOS and/or by scavenging formed peroxynitrite in the gastric mucosal tissue.

The results of the present study indicate that, in rats with a single compound 48/80 treatment, ebselen administered orally at the stage of gastric mucosal lesion formation exerts a protective effect against the lesion progression, and suggest that this protective effect of ebselen could be due to its GSH-px-like activity and its antioxidative and anti-inflammatory properties.

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